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APPLICATION NUMBER: ***60/462,364***
FILING DATE: ***April 11, 2003***
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PTO/SB/16 (10-01)
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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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TITLE OF THE INVENTION (500 characters max)

METHODS FOR PREPARATION OF SITE-SPECIFIC PROTEIN CONJUGATES

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ENCLOSED APPLICATION PARTS (check all that apply)

- [X] Specification Number of Pages 23
- [X] Drawing(s) Number of Sheets 3
- [X] Application Data Sheet. See 37 CFR 1.76
- [ ] CD(s), Number
- [ ] Other:

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

- [X] Applicant claims small entity status. See 37 CFR 1.27.
- [ ] A check or money order is enclosed to cover the filing fees
- [X] The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: 12-0080

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- [ ] Payment by credit card. Form PTO-2038 is attached.

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

[X] No [ ] Yes, the name of the U.S. Government agency and the Government contract number are:

Respectfully submitted,

SIGNATURE Jane Remillard

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Date April 11, 2003

REGISTRATION NO. (if appropriate) 38,872

Docket Number: PRJ-013-1

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

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Dated: April 11, 2003 Signature: Jane Remillard (Jane E. Remillard, Esquire)

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PROVISIONAL APPLICATION COVER SHEET
Additional Page

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Docket Number	PRJ-013-1

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FEE TRANSMITTAL for FY 2003

Patent fees are subject to annual revision.

[X] Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 80.00

Complete if Known	
Application Number	Not Yet Assigned
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First Named Inventor	Kenenth Hinds
Examiner Name	Not Yet Assigned
Group Art Unit	N/A
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[ ] Check [ ] Credit Card [ ] Money Order [ ] Other [ ] None

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Deposit Account Number: 12-0080

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The Commissioner is hereby authorized to: *(check all that apply)*

[X] Charge fee(s) indicated below [X] Credit any overpayments

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FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1001	750	2001	375	Utility filing fee	
1002	330	2002	165	Design filing fee	
1003	520	2003	260	Plant filing fee	
1004	750	2004	375	Reissue filing fee	
1005	160	2005	80	Provisional filing fee	80.00

SUBTOTAL (1) ($) 80.00

2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

			Extra Claims		Fee from below	=	Fee Paid
Total Claims		-** =		x		=	
Independent Claims		-** =		x		=	
Multiple Dependent						=	

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
1202	18	2202	9	Claims in excess of 20
1201	84	2201	42	Independent claims in excess of 3
1203	280	2203	140	Multiple dependent claim, if not paid
1204	84	2204	42	** Reissue independent claims over original patent
1205	18	2205	9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($) 0.00

**or number previously paid, if greater; For Reissues, see above

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1051	130	2051	65	Surcharge – late filing fee or oath	
1052	50	2052	25	Surcharge – late provisional filing fee or cover sheet.	
1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for *ex parte* reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	
1252	410	2252	205	Extension for reply within second month	
1253	930	2253	465	Extension for reply within third month	
1254	1,450	2254	725	Extension for reply within fourth month	
1255	1,970	2255	985	Extension for reply within fifth month	
1401	320	2401	160	Notice of Appeal	
1402	320	2402	160	Filing a brief in support of an appeal	
1403	280	2403	140	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive – unavoidable	
1453	1,300	2453	650	Petition to revive - unintentional	
1501	1,300	2501	650	Utility issue fee (or reissue)	
1502	470	2502	235	Design issue fee	
1503	630	2503	315	Plant issue fee	
1460	130	1460	130	Petitions to the Commissioner	
1807	50	1807	50	Processing fee under 37 CFR 1.17(q)	
1806	180	1806	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
1809	750	2809	375	Filing a submission after final rejection (37 CFR 1.129(a))	
1810	750	2810	375	For each additional invention to be examined (37CFR 1.129(b))	
1801	750	2801	375	Request for Continued Examination (RCE)	
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Other fee (specify)					

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Dated: April 11, 2003 Signature: (Jane E. Remillard, Esquire)

Application Data Sheet

Application Information

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METHOD FOR PREPARATION OF SITE-SPECIFIC PROTEIN CONJUGATES

Field of the Invention

The invention relates to chemically modified protein conjugates that possess superior properties to those of the unconjugated protein. More specifically the invention relates to a greatly simplified, cost effective and process scalable method for the modification of proteins with hydrophilic polymers. More specifically the invention relates to the site-specific modification of selected proteins, such as insulin, with poly(ethylene glycol).

Description of the Related Art

A variety of methods for producing PEGylated insulin derivatives are known. Davis et al. (U.S. Patent No. 4,179,337) described the synthesis of a PEG-insulin construct using trichloro-s-triazine (cyanuric chloride) as the linker between PEG and protein. They followed a synthetic scheme in which a large excess (50X) of cyanuric chloride activated PEG (2000 Da) was reacted with insulin in borate buffer (pH 9.2) for 2hr. The inventors were able to produce partially active (~50%) PEG-insulin conjugates, which were non-immunogenic and non-antigenic. Obermeier et al. (Canadian Patent No. 1,156,217), found that preparation of PEG-insulin conjugates according to Example X of the Davis patent referenced above produced a non-uniform mixture of conjugates containing approximately 50% tri-PEG-insulin, and the other possible PEG-insulin derivative combinations (mono- and di-PEG-insulins) were not substituted at residue PheB1.

Obermeier et al. (Canadian Patent No. 1,156,217) describe a synthesis of PEG-insulin conjugates specifically modified at residue PheB1. The basis of their invention involves protecting the reactive amines at residues GlyA1 and LysB29 with tert-butyloxycarbonyl (t-boc) or methylsulfonylethyloxycarbonyl (Msc) groups in organic solvents (e.g., DMF, DMSO, pyridine, etc.) under alkaline conditions. From the complex mixture of (mono-, di-, and tri-) protected insulins the $N^{\alpha A1}$, $N^{\varepsilon B29}$-bis-protected-insulin species was isolated by conventional chromatographic techniques. Following isolation, the pure $N^{\alpha A1}$, $N^{\varepsilon B29}$-bis-protected-insulin was reacted with an activated (e.g., acid chloride or isocyanate) PEG derivative with subsequent removal

of the protecting groups using techniques common to peptide chemistry. The inventors observed that the amino groups of GlyA1 and LysB29 were more reactive than PheB1's amino group under alkaline reaction conditions. They determined their site-specific mPEG(1500)-B1-insulin conjugates had a 100% insulin effect (calculated on a molar basis) on reduction of blood sugar levels in rabbits.

Geiger et al. (in D. Branderburg, and A. Wollmer (eds.), Insulin: Chemistry, Structure, and Function of Insulin and Related Hormones, Walter de Gruyter & Co., New York, pp. 409-415, 1980) and Ehrat et al. (Biopolymers, 22, 569-573, 1983) describe PEG-insulin adducts specifically modified at residue PheB1 prepared utilizing a protection/conjugation/deprotection scheme similar to the multi-step method described by Obermeier et al. Geiger et al. and Ehrat et al. observed that the PEG(1500)-B1-insulin conjugate was far less antigenic and far more stable (to liver enzymes) than native insulin. Other PEG-insulin preparations (Caliceti et al., STP Pharma Sci, 9, 107-113, 1999; Uchio et al., Advanced Drug Delivery Reviews, 35, 289-306, 1999; Hinds et al., Bioconj. Chem. 11, 195-201, 2000; Hinds et al., Advanced Drug Delivery Reviews, 54: 505-530, 2002) are either: 1) centered on the basic three-step protection/conjugation/deprotection schemes outlined above, 2) result in non-specific modification of the insulin molecule, or 3) do not produce the most effective conjugates, namely, PEG-B1-insulins.

Liu et al. (US Patent No. 6,323,311 B1) describe a useful method for the synthesis of PEG-B1-insulin conjugates. This method is an extension of the Obermeier three-step protection/conjugation/deprotection scheme, but does not require the isolation of reaction intermediates between steps (i.e., one-pot synthesis). Thus, the insulin is protected at residues GlyA1 and LysB29, immediately reacted with PEG, and subsequently deprotected before any isolation of species. The inventors claim that their one-pot reaction may yield up to 50% of the correct positional isomer (i.e. PEG-B1-insulin) and 30% unreacted insulin that can be recycled for subsequent derivatization. Assuming the preparation of these constructs in carried out expeditiously, it would take at least five days to completion. In addition, the invention requires large excesses of the PEG reagent to achieve acceptable results. While the products of this invention may be effective, their preparation still requires the protein to undergo three reaction steps in protein-adverse environments (high and low pH) for extended periods of time.

The present invention addresses the shortcomings of prior art methods for PEGylating insulins by providing a method for the simple preparation of highly pure insulin derivatives specifically PEGylated at the N-terminus of insulin's B-chain (PheB1) in a single-step. In contrast to prior experience (e.g., Caliceti et al., 1999, supra) indicating that PEGylation at PheB1 is the least probable reaction product, the present method employs specific conditions of pH control, use of a metal ion chelator and addition of organic solvent to enhance the relative reactivity of the PheB1 amino terminus to where it becomes the predominant site of PEGylation. Considering the numerous beneficial properties imparted on insulin (e.g., decreased immunogenicity/antigenicity; increased proteolytic, chemical and physical stability; increased circulation half-life; increased aqueous/organic solubility; full biological activity) via site-specific PEGylation at residue PheB1, a simple, cost-effective, and easily scalable process for achieving this result would be a significant advancement in the art.

SUMMARY OF THE INVENTION

The present invention is based on the discovery of a single-step method for preparing protein-polymer conjugates. In a particular embodiment, the present invention provides a single-step method for synthesis of PEGylated insulin derivatives wherein the site of substitution is predominantly residue PheB1 (N-terminus of the B-chain). It is well known in the art that such derivatives are physically and enzymatically more stable than native insulin. In addition, the derivatives are more soluble in aqueous/organic systems than insulin. Moreover, these derivatives have been shown to be less immunogenic and to have prolonged circulation half-lives.

A significant advantage of the present invention is that the reaction takes place in near-neutral conditions where unwanted side-reactions (e.g., deamidation, transamidation, oxidation, etc.) are negligible. Another advantage is the use of relatively low amounts of polymer (e.g., PEG reagent), thus reducing costs. The resulting protein-polymer conjugate (e.g., PEGylated insulin) may be used by itself, for example, in human therapy, or it may be encapsulated in a sustained release delivery construct, such as disclosed in, e.g., U.S. Patent Application 2002/0155158.

Accordingly, in one embodiment, the present invention provides a method for preparing a protein-polymer conjugate by contacting an insulin protein with a hydrophilic polymer in the presence of at least one organic solvent and at least one

metal chelator, such that (i.e., under conditions whereby) a conjugate of the protein and polymer is formed. The conjugate can then be isolated using a variety of standard techniques, such as column chromatography.

In a particular embodiment of the invention, the hydrophilic polymer is selected from the group consisting of polyethylene glycol, polypropylene glycol, and linear, branched and amino-reactive derivatives thereof. Suitable amino-reactive derivatives include, for example, aldehydes, N-hydroxy succinimides, PNP-carbonates, and benzotrizole terminated hydrophilic polymer derivatives. Typically, the hydrophilic polymer and insulin protein are present at a molar ratio of about 10:1-1:1.

Suitable organic solvents for use in the invention include a wide variety of known solvents including, but not limited to, water-miscible organic solvents, such as ethanol, methanol, DMSO, dioxane, DMF and NMP. Typically, the organic solvent is present at a concentration of about 0.1 to 10 %.

Suitable metal chelators for use in the invention also include a wide variety of known compounds including, but not limited to, polyvalent (e.g., divalent) metal ion chelators, such as EDTA, deferoxamine (DEF), diethylenetriamine pentaacetic acid (DTPA), and bis(aminoethyl)glycolether N,N,N',N'–tetraacetic acid (EGTA). Generally, the chelator is present at a concentration from about 0.1-10 mM.

In a particular embodiment of the invention, the insulin protein and hydrophilic polymer (e.g., PEG) are contacted (i.e., reacted or conjugated) in an aqueous solution at a protein concentration of about 0.1-5% by weight. In another embodiment of the invention, the insulin protein and hydrophilic polymer are contacted in an aqueous solution at a pH of about 5.0-7.5, preferably about 7.0. This results in a yield of up to 50% of the correct positional isomer of insulin-polymer conjugate.

Once formed, the protein-polymer conjugate is then separated from unwanted side reaction products and unreacted insulin protein. This can be achieved using a variety of known techniques, such as chromatography. In a particular embodiment, ion exchange chromatography is employed.

In yet another embodiment, the method of the present invention further comprises the step of quenching the reaction (i.e., conjugation) of insulin protein and hydrophilic polymer, prior to isolating the conjugation product. In a particular embodiment, this is achieved by reducing the pH of the reaction to about 1-4.

Particular protein-polymer conjugates produced by the methods of the present invention include, for example, insulin-polymer conjugates, preferably, insulin-PEG conjugates (PEGylated insulin). This can include any insulin or insulin-related protein, such as human insulin and related family members. In a particular embodiment, the insulin is specifically reacted (PEGylated) at residue PheB1, without significant reaction at residues GlyA1 or LysB29. The resulting PEGylated insulin can be administered therapeutically in any suitable formulation as is well known in the art. In a particular embodiment, the conjugate is administered in a sustained release formulation by, for example, encapsulating the conjugate in a biodegradable polymer prior to administration.

Other embodiments of the present invention will be apparent from the following Detailed Description and Examples 1-8.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph showing in vitro release of F5000 from microspheres.

Figure 2 is a graph showing in vivo pharmacodynamics of F5000 delivered from PLGA microspheres in diabetic rats.

Figure 3 is a graph showing in vivo pharmacokinetics of F5000 delivered from PLGA microspheres in diabetic rats.

DETAILED DESCRIPTION

The present invention is directed to a single-step method for rapidly and efficiently preparing protein-polymer conjugates. The method includes reacting a protein and a hydrophilic polymer in the presence of at least one organic solvent and at least one metal chelator, under near-neutral conditions. Particular protein-polymer conjugates of the invention include insulin, PEGylated at the PheB1 amino terminus using minimum PEG reagent and mild conditions. Whereas prior work has shown that PheB1 is normally the least reactive amino group of insulin (see, e.g., Caliceti et al., 1999, supra), surprisingly, the methods of the present invention produce conditions in which PheB1 is the most reactive. This allows for a simple, one-step reaction in which insulin PEGylated at PheB1 is the highest yield product, and can be separated from the other conjugates and unreacted insulin. The latter may be recycled for further conjugation if desired. The PEGylated PheB1 insulin conjugate retains full

activity as measured by blood glucose control and the protein native structure is preserved.

Hydrophilic Polymers

The term "hydrophilic polymer" refers to any water-soluble linear or branched polymer including, but not limited to, polyethylene glycol and polypropylene glycol and similar linear and branched polymers. Preferably, the molecular weight of the polymer ranges from about 500 daltons to about 50,000 daltons. Hydrophilic polymers for use in the invention typically have a reactive group incorporated for attachment to the bioactive molecule of interest through amino, carboxyl, sulfhydryl, phosphate or hydroxyl functions.

Hydrophilic polymers used in the present invention, such as polyethylene glycol, can be prepared according to standard protocols with one end capped as with a methoxy group and the other end activated for facile conjugation to active groups on bioactive molecules. For example, U.S. Patent No. 6,113,906 describes the use of succinamidyl succinate or carbamate reactive groups on the polyethylene glycol to react with amine groups on proteins. U.S. Patent No. 5,446,090 describes the use of sulfone derivatives of polyethylene glycol to form stable bonds with sulfhydryl groups of proteins. U.S. Patent No. 5,880,255 describes the use of tresyl derivatives for reaction at amine groups of proteins to form a simple, stable secondary amine linkage. The entire contents of these patents is incorporated by reference herein. N-hydroxy succinamide also may be incorporated as the reactive group.

In one embodiment, the hydrophilic polymer is poly (ethylene glycol) (PEG). PEG refers to a linear or branched neutral polyether with the chemical formula HO-$(CH_2CH_2O)_n$-H. PEG is highly soluble in water and many organic solvents (e.g., methylene chloride, ethanol, toluene, acetone, and chloroform), and is readily available in various sizes (molecular weights) and functionalized architectures (e.g., amino-, carboxyl-, and sulfhydryl-terminated). PEG has been found to be nontoxic and is approved by the FDA for use in drugs (parenterals, topicals, suppositories, nasal sprays), foods, and cosmetics. In solution PEG is a highly hydrated polymer, where each monomer (ethylene oxide unit) can bind up to three molecules of water. In addition, it is thought that PEG has the ability to influence the structure of several molecular layers of more loosely associated hydrating water molecules. Molecular simulations of the behavior of single surface-bound chains in water show the polymer

exhibits a large degree of segmental flexibility. Thus, the polymer is assumed to occupy a large hydrodynamic volume in aqueous environments. These findings serve to explain why PEG is remarkably effective in excluding other polymers (natural and synthetic) from its presence. The exclusion of other polymers is the primary driving force behind PEG's ability to reject proteins, form two-phase systems with other synthetic polymers, and makes this polymer both nonimmunogenic and nonantigenic. When PEG is covalently attached to a protein, it typically transfers many of the polymer's favorable characteristics to the resultant conjugate. Because of the many beneficial properties mentioned above, PEG is well suited for protein modification.

As used herein, the term "PEG" includes any PEG protein, including amino-reactive derivatives of PEG ("PEG reagents"). A variety of PEG reagents for protein conjugation are known. A typical PEG reagent is a linear PEG polymer with one end terminated in an ether linkage (e.g. O-methyl) and the other end functionalized with a reactive group. Other PEG reagents are branched or dendrimeric, again with a combination of non-reactive termini and reactive functional groups for linking to proteins. Examples of PEG reagents include, but are not limited to, an aldehyde, a N-hydroxy succinimide, a PNP-carbonate, or a benzotriazole terminated species or other amino-reactive activated species of PEG.

The PEG polymer may have a molecular weight in the range of, for example, 500 to 50,000 Da. The reactive functional groups may be separated from the PEG chain by linker groups. Optionally, the polymers have degradable internal bonds between the PEG and linkers. Accordingly, in one embodiment of the invention, reactive groups on the PEG polymer may be electrophilically activated for reaction with protein nucleophiles. Examples of electrophilic groups are n-hydroxy succinamide, tresyl, and aldehyde functionalities. PEG reagents with these functionalities will react to form covalent linkages to amino groups of proteins. A preferred PEG reagent for conjugation to protein amino groups is monomethoxy PEG N-hydroxy succinamide (mPEG-NHS). Another preferred PEG reagent is the mPEG succinimidyl active ester of a propionic acid linker mPEG-SPA.

Insulin-Polymer Conjugates

Protein PEGylation has been used to improve the therapeutic efficacy of recombinant human proteins. Most parenterally administered proteins are rapidly cleared from the body by the reticuloendothelial system (RES), kidney, spleen, and

liver. Moreover, clearance depends on molecular size, charge, and the presence of specific cellular receptors for the proteins of interest. The attachment of PEG to a protein affects its molecular size, charge, and receptor-binding capabilities, which can serve to decrease the conjugate's overall rate of clearance.

In addition, the metabolism of proteins by enzymes leads to the rapid loss of biological activity of therapeutic proteins. By sterically shielding the protein domains susceptible to proteolytic attack, PEG decreases the protein degradation that renders it biologically inactive.

Moreover, even recombinant human proteins elicit immune responses after repeated use. By sterically masking the therapeutic protein's immunogenic/antigenic determinants, PEG-attachment commonly results in conjugates that are nonimmunogenic and nonantigenic. Accordingly, overall, the result of changes in the parental protein's characteristics by PEGylation increases the plasma half-life and resistance to proteolytic degradation and decreases immunogenicity and antigenicity of the resultant PEG-protein construct.

Insulin Protein

The term "insulin protein," as used herein, refers to any naturally occurring or recombinant insulin protein or related protein capable of being conjugated at, for example, residue PheB1. Accordingly, insulin proteins for use in the invention include, for example, insulin analogs, homologs and derivatives. Insulin from any suitable species can be used, such as human, pig, cow, dog, rat, mouse, sheep, or monkey. In a preferred embodiment, the insulin is human insulin.

Human insulin is a well-known protein, which is readily available commercially from a number of sources including, but not limited to, Sigma Chemical Company and Novo Nordisk. Naturally occurring human insulin is a protein having a molecular weight of approximately 5,500 daltons and includes approximately 51 amino acids. Depending on the manufacturer, the insulin may have slightly different activity based upon weight, however, the activity of insulin defined in units is the standard. Insulin having various degrees of biological activity is commercially available. For instance, it is possible to purchase low-, intermediate-, and rapid-acting forms of insulin. Non-insulin hypoglycemic agents that have similar activities to insulin or which increase insulin receptors include, but are not limited to, sulfonyl ureas (e.g., glibenclamide, gliclazide, glipizide, glyburide, chlorpropamide,

tolbutamide, tolazamide, acetohexamide, and glimopride); thiazolidine diones (e.g., troglitazone and ploglitazone); alpha.-glucosidase inhibitors (e.g., acarbose and miglitol); and third generation insulin-releasing agents (e.g., KAD 1220, etoxomir, and repaglinide).

The insulin molecule consists of two polypeptide chains, the A-chain and the B-chain. The A-chain is composed of 21 amino acids (denoted A1-A21), and the longer B-chain is comprised of 30 amino acids (B1-B30). These two chains are held together by two inter-chain disulfide bonds between residues A7 and B7, as well as, between A20 and B19, whereas another intra-chain disulfide bond connects residues A6 and A11. There are also numerous noncovalent interactions between the amino acid residues of the two chains that help to stabilize insulin into its three-dimensional structure.

Insulin proteins also include related proteins, such as the insulin-like growth factors (I and II), and proteins from the growth hormone/prolactin family.

There are three reactive amino groups available for modification (e.g., by PEG) namely, those located at the A- and B-chain N-termini (A1 and B1, respectively) and a lysine located adjacent to the B-chain C-terminus (B29).

Conjugation of Insulin Protein to Hydrophilic Polymer

In accordance with the present invention, the insulin protein and hydrophilic polymer are contacted (i.e., reacted or conjugated) in the presence of at least one organic solvent and at least one metal chelator, under conditions that promote the formation of a conjugate of the protein and polymer. In a particular embodiment, the insulin protein is PEGylated at the PheB1 amino terminus using minimum PEG reagent and mild conditions. The amino group of PheB1 is normally the least reactive of the 3 available amino functions on insulin (Caliceti et al., 1999, supra). In the present invention, conditions have been found that render the PheB1 amino group the most reactive to PEG reagents. These reaction conditions thus produce single PEGylation at the PheB1 as the predominant reaction product.

In a particular embodiment of the present invention, the insulin protein and hydrophilic polymer are contacted in an aqueous solution at a protein concentration of about 0.1 to 5% (w/w), preferably from 0.5 – 1.5%, adjusted to a pH in the range 5.0 to 7.5, preferably pH 6.5 to 7.2. The pH can be controlled by inclusion of buffer salts,

addition of organic acids/bases, or addition of common inorganic acids/bases. The aqueous solution further comprises at least one water miscible organic solvent, which may be are selected from the group including ethanol, methanol, DMSO, dioxane, DMF, NMP, etc. In another aspect, the organic solvent, preferably dioxane, is included at a concentration (v/v) of from 0 to 25%, preferably from 2 – 20%, more preferably from 5-15%.

Suitable metal chelators for use in the invention include a wide variety of known chelators including, for example, aminopolycarboxylic acids, such as, ethylenediaminetetraacetic acid (EDTA), diethylenetriamine pentaacetic acid (DTPA), nitrilotriacetic acid (NTA), N-2-acetamido-2-iminodiacetic acid (ADA), bis(aminoethyl)glycolether, N,N,N',N'-tetraacetic acid (EGTA), trans-diaminocyclohexane tetraacetic acid (DCTA), glutamic acid, and aspartic acid; and hydroxyaminocarboxylic acids, such as, for example, N-hydroxyethyliminodiacetic acid (HIMDA), N,N-bis-hydroxyethylglycine (bicine) and N-(trishydroxymethylmethyl) glycine (tricine); and N-substituted glycines such as glycylglycine. Other suitable chelators include 2-(2-amino-2-oxocthyl) aminoethane sulfonic acid (BES) and deferoxamine (DEF). Suitable chelators used in the methods of the present invention include, for example, those that bind to metal ions in solution to render them unable to react with available O_2, thereby minimizing or preventing generation of ·OH radicals which are free to react with and degrade the protein. Such chelators can reduce or prevent degradation of a protein that is formulated without the protection of a chelating agent.

Chelating agents used in the invention can be present in their salt form *e.g.*, carboxyl or other acidic functionalities of the foregoing chelators. Examples of such salts include salts formed with sodium, potassium, calcium, and other weakly bound metal ions. As is known in the art, the nature of the salt and the number of charges to be neutralized will depend on the number of carboxyl groups present and the pH at which the stabilizing chelator is supplied. As is also known in the art, chelating agents have varying strengths with which particular target ions are bound. In general, heavy metal ions are bound more strongly than their similarly charged lower molecular weight counterparts.

The chelator used in the methods of the present invention may also be selected from EDTA, EGTA, and other multivalent cation chelators known in the art. According to the methods of the invention, a metal chelator, preferably EDTA, is

present at a concentration from 0.1 to 10 mM, preferably from 1 – 5 mM, more preferably from 1-3 mM.

Suitable hydrophilic polymers for use in the present invention include a wide variety of known polymers including, for example, polyethylene glycol, polypropylene glycol, and linear, branched and amino-reactive derivatives thereof. In one aspect of the invention, the amino-reactive derivative is selected from the group consisting of an aldehyde, a N-hydroxy succinimide, a PNP-carbonate, and a benzotriazole terminated hydrophilic polymer derivative. In a particular embodiment of the invention, the hydrophilic polymer, e.g. a PEG reagent, preferably a succinimidyl PEG, more preferably mPEG-SPA, is contacted with insulin at a molar ratio (PEG:insulin) of about 10:1 to 1:1, preferably 3:1 to 1.2:1, more preferably 1.7:1 to 1.5:1. In another particular embodiment, the hydrophilic polymer and insulin protein are contacted at a temperature of about 4°C to 50°C, preferably about 15°C to 25°C.

In another embodiment, the invention further comprises the step of quenching the conjugation reaction prior to isolating the conjugate. This can be achieved by, for example, reducing the pH to about 1-4, preferably about 2 – 3, more preferably about 2.4 to 2.6. The isolation of the conjugate can then be achieved using standard techniques, such as ion exchange (e.g., cation exchange) chromatography, and the desired conjugate may be collected, concentrated, desalted and dried.

Use of Conjugated Bioactive Agents in Controlled Release Delivery Formulations

Conjugated bioactive agents, such as PEGylated insulin proteins, may be advantageously encapsulated in biodegradable polymer-based drug delivery formulations as is well known in the art. PEGylated bioactive agents are encapsulated at higher concentration in the drug delivery formulation than the corresponding non-PEGylated bioactive agents. The release of PEGylated bioactive agents from biodegradable polymer drug delivery formulations shows less-burst than for the corresponding non-PEGylated bioactive agents. The physical and chemical stability of PEGylated bioactive agents in biodegradable polymer drug delivery formulations is greater, and the antigenicity and immunogenicity are lower than for the corresponding non-PEGylated bioactive agents.

Biodegradable polymers for this application include, but are not limited to, poly(lactide)s, poly(glycolide)s, poly(d,l-lactide-co-glycolide)s, poly(caprolactone)s,

poly(orthoester)s, copolymers of poly(esters) and poly(ethers), copolymers of poly(lactide) and poly(ethylene glycol) and the like.

Accordingly, protein-polymer (e.g., PEGylated insulin) conjugates of the present invention can be beneficially incorporated into biodegradable polymer drug delivery formulations including, for example, poly(d,l-lactide-co-glycolide) (PLGA) microparticles. This achieves higher encapsulation of the protein conjugate as compared to non-conjugated protein and also reduces burst (release over the first 24 hours). Moreover, conjugation with hydrophilic polymers, such as PEG, renders the conjugate soluble in certain organic solvents, simplifying the process of forming PLGA microspheres.

EXAMPLES

I. PREPARATION AND CHARACTERIZATION OF SITE-SPECIFIC PEG-INSULIN BY ONE-STEP METHOD

EXAMPLE 1

Preparation of $N^{\alpha B1}$-methoxypoly (Ethylene Glycol)-insulin conjugates.

One gram (172 μmol) of insulin (Zn^{2+}-insulin, Intergen Co.) was dissolved in 100mL water with 2mM EDTA at room temperature, and the solution's pH_{app} was adjusted to 7 using dilute HCl. In another vessel 1.4g (1.6 mol equivalents relative to insulin) of an activated PEG derivative [monomethoxypoly (ethylene glycol) succinimidyl propionate, mPEG-SPA, Shearwater Corp.] was dissolved in 10mL dioxane at room temperature. The mPEG-SPA solution was then added to the insulin solution by direct injection and the reaction was allowed to proceed for 2 hr at room temperature. The reaction was then quenched by acidification with HCl (pH_{app} 2.5) and the mixture was diafiltered [Amicon 8200 ultrafiltration apparatus fitted with a YM3 (3000 MWCO) membrane] against 0.02% ammonium bicarbonate. Then the reaction mixture was diafiltered against 1M acetic acid/7M urea/0.01M NaCl and concentrated to 10mL prior to purification. The mPEG-PheB1-insulin derivative was isolated from the other reaction side-products (mPEG-GlyA1-insulin, mPEG-LysB29-insulin, di-mPEG-insulins, and tri-mPEG-insulins) using a FPLC system fitted with a SP Sepharose (Amersham Biosciences) cation-exchange column. The column was equilibrated with 1M acetic acid/7M urea containing 0.04M NaCl at a flow rate of 5

mL/min and the bound proteins were eluted using a NaCl-gradient (0.04M-0.12M) over 80 min. The eluate corresponding to the major peaks detected at 280nm were collected and diafiltered against 0.02% NH_4HCO_3 to remove any low molecular weight impurities, then lyophilized and stored at -20°C prior to characterization

EXAMPLE 2

FPLC/HPLC assessment of conjugate purity.

The purity of the mPEG-PheB1-insulin was analyzed using an analytical cation-exchange column (MonoS 5/5, Amersham Biosciences) under identical conditions to those used in the isolation procedure described above, except a flow rate of 1.0 mL/min was employed. An orthogonal technique (reversed-phase HPLC) was also used to verify the final purity of the conjugates. A Waters Alliance HPLC system was fitted with a Waters 996 photodiode array detector (PDA) and a Symmetry 300 (C18, 5µm particle size, 4.6x250mm) reverse phase column. Mobile phase A consisted of 0.1% TFA (trifluoroacetic acid) in MilliQ quality water and mobile phase B consisted of 95/5 ACN (acetonitrile)/H_2O also containing 0.1%TFA. A linear gradient from 30-60% B over 15 min (2%B/min) was utilized and the elution of compounds was followed by detection at 276nm. The purity of the mPEG-insulin is >95%.

EXAMPLE 3

N-terminal protein sequencing (Edman degradation) confirmation of conjugate identity.

N-terminal protein sequence analysis was utilized to determine the site of PEG conjugation, with the knowledge that the Edman degradation reaction will not proceed at any N-terminal amino group that is covalently bound to PEG. All samples were analyzed using an Applied Biosystems 477A Protein Sequencer (Pasadena, CA) through three Edman degradation cycles. An N-terminal amino acid molar ratio of [GlyA1/PheB1] $\approx$ 1 is indicative of conjugation to residue LysB29 (or none at all), a [GlyA1/PheB1] $\approx$ 0 is indicative of conjugation to residue GlyA1, and [GlyA1/PheB1] $\approx$ 30 is indicative of conjugation to residue PheB1. The result confirmed that the site of substitution was approximately 95% PheB1

EXAMPLE 4

Matrix-assisted laser desorption ionization (MALDI) identification of conjugate molecular weight.

This analytical characterization technique was chosen because it is a "soft-ionization" method meaning that it will not cause the PEG-insulin conjugates to break down during analysis. The instrument's output provides a quantitative measure of the mass/charge ratio of each sample; therefore the number of PEG chains attached to insulin should be easily determined from the overall difference in the molecular weights of the conjugates and unmodified insulin. All samples were run on a Perceptive Biosystems model DERP MALDI/TOF mass spectrometer operated in the linear mode and positive ions were monitored. The matrix for all samples was α-cyano-4-hydroxycinnamic acid and the 337 nm line of a nitrogen laser was used with at least 64 shots averaged for the final spectrum. Monomeric insulin had a calculated molecular weight of 5807.2 Da, and the number-average molecular weight of mPEG(5000)-SPA used in the conjugation reaction was 5129 Da. The mass spectra of the mPEG(5000)-insulin were consistent with the conclusion that only one mPEG chain was attached to insulin. In addition, the individual ion peaks consistently differed by 44 Da (the molecular weight of an ethylene oxide monomer unit). These results confirm that only one mPEG chain was attached to insulin in all of the conjugates prepared and that their polydispersity is solely due to the polydispersity intrinsic to PEG.

EXAMPLE 5

Secondary Amine Formation in Coupling PEG to Insulin B1 Amino Terminus.

F5000 PEG-insulin was prepared by reaction of mPEG activated with an aldehyde functionality to form a Schiff's base which is reduced by cyanoborohydride to a secondry amine. The reactions were carried out as follows: a 2mM EDTA / 25mM phosphate buffer was made and adjusted the pH to 6.0 with phosphoric acid. Insulin at 5.5mg/mL (2mL total volume) was dissolved in the phosphate buffer with the addition of 440μL dioxane. Once the insulin was in solution 2 mL of a 10mM $NaCNBH_3$ solution in water were added and then a 5x molar excess of mPEG(5000)-aldehyde (as a dry powder, Shearwater Corporation, Huntsville, Alabama) was added. Overall, the reaction mixture contained approximately 12.5mM phosphate (pH 6.),

1mM EDTA, 10% dioxane, 5mM $NaCNBH_3$, 2.5mg/mL insulin, and 10mg/mL mPEG-aldehyde. The reaction was allowed to proceed overnight and the pH was found to be approx. 5.5 the following day. The reaction was quenched with addition of acetic acid to a pH of approximately 2. A small aliquot was analyzed using RP-HPLC. The major reaction species were determined as follows: Approximately 70% of the reaction products are mono-PEGylated (r.t. 12.5 min.), with approximately 10% unreacted insulin (r.t. 9.8 min.) and 9% di-PEGylated (r.t. 13.8 min) insulin remaining. The mono-PEGylated fractions were pooled and dialyzed against 0.02% NH_4HCO_3 and lyophilized. MALDI-TOF analysis showed a single molecular mass corresponding to addition of one PEG-5000 chain to insulin. Edman degradation analysis showed that approximately 95% of the mono-PEGylated species were substituted at residue PheB1 and the remaining mono-PEGylated species (~5%) substituted at residue GlyA1.

II. ALTERNATIVE PREPARATION OF SITE SPECIFIC PEG-INSULIN

EXAMPLE 6

PEG-5000 linked to B chain amino terminus (F5000).

Recombinant human insulin (Intergen Co.) was PEGylated at the PheB1 position utilizing a di-boc protected intermediate. Di- $N^{\alpha A1}$, $N^{\varepsilon B29}$-*t*-boc-insulin (diboc-insulin) was synthesized according to Liu et al. 1997 (Liu et al., Bioconj. Chem. 8(5):664-672, 1997). The mPEG (5000)-PheB1-insulin conjugate was prepared by the protocol of Hinds et al., 2000, supra.

The desired fraction from FPLC was > 98% pure based on chromatographic peak areas in reverse-phase HPLC and ion exchange FPLC. The purified product was further characterized by MALDI-TOF mass spectrometry and amino acid sequence analysis and was shown to be mono-substituted PEG at the B chain amino terminus, PheB1. The mPEG(5000)-PheB1-insulin conjugate (F5000) prepared and purified by this method was equivalent to the same conjugate made by the simpler and less time-consuming method of the present invention (Example 1).

PEG-5000 linked to B chain Lys29 (K5000).

Recombinant human insulin (Intergen Co.) was PEGylated specifically at Lys29 of the B chain by the method of Hinds et al., 2000, supra. The desired FPLC fraction was > 98% pure based on chromatographic peak areas in reverse-phase HPLC and ion exchange FPLC. The purified product was further characterized by MALDI-TOF mass spectrometry and amino acid sequence analysis and was shown to be mono-substituted PEG at the B chain penultimate amino acid, LysB29.

Assessment of insulin conformational integrity following site-specific PEGylation.

Circular dichroism spectroscopy in the far-ultraviolet range has been used to examine the conformation of insulin. Usually the magnitudes of two negative minima are evaluated: 208 nm (α-helix) and 223 nm (β-sheet) in the analysis of insulin's conformation in aqueous environments. The far ultraviolet CD-band at 208 nm primarily arises from α-helicies formed from residues between B10-B19, A2-A6, and A13-19, while β-structure is the primary component of the far ultraviolet CD-band at 223 nm. The CD spectral characteristics of the samples confirm that attachment of mPEG to insulin at either residue PheB1 or LysB29, does not alter the overall conformation (secondary) of the conjugates as compared to Zn^{2+}-insulin.

EXAMPLE 7

Pharmacodynamics of F5000 and K5000 PEG-Insulins.

Blood glucose levels were measured for fasted Sprague-Dawley rats after intravenous administration of F5000, K5000 and Humulin®. The equivalent of 0.3IU/kg (based on weight and corrected for the weight of the PEG as appropriate; assumes 25 IU/mg protein) were dissolved in normal saline and administered by tail vein injection; N=6 per group. Blood was drawn before test article injection and at intervals over a period of 6 hrs post injection. Serum was isolated by standard procedure. Serum glucose levels were measured using an Accucheck Advantage (Boeringer Ingelheim) glucometer.

Results showed that F5000 and K5000 are as active as equivalent doses of normal human insulin to suppress glucose levels in normal rats.

III. PREPARATION, CHARACTERIZATION, AND PHARMACOKINETICS OF PEG-INSULIN BIODEGRADABLE POLYMER DELIVERY FORMULATIONS

EXAMPLE 8

Encapsulation of PEG-Insulin in PLGA Microspheres.

PEG-insulin F5000 (50 mg) was dissolved in 1 ml methylene chloride. The solution was added to a volume of 1 ml methylene chloride containing 150 mg PLGA 45:55 (lac:gly), 0.15dl/g IV with acid end groups. The methylene chloride solution was added to 5 ml water containing 1% poly(vinyl alcohol) in a 15 ml centrifuge tube and vortex-mixed to form an emulsion. The emulsion was added to 100ml of water containing 0.3% poly(vinyl alcohol) and stirred for 3 hrs to evaporate the methylene chloride. The hardened microspheres were collected by vacuum filtration on Whatman #1 filter paper and dried. Drug content was determined by dissolving a measured quantity of microspheres in 50% acetonitrile and analyzing by reverse phase HPLC. Morphology was examined by scanning electron microscope.

K5000 was encapsulated by the same method, and other polymers and drug loadings were tested with each PEG-insulin. The results of these tests are collected in Table 5, which lists the PEG-insulin content in %(w/w) of the total microsphere and the encapsulation efficiency, defined as weight of PEG-insulin encapsulated/weight of PEG-insulin added initially. Relatively high drug content up to 28.3% and encapsulation efficiency, up to 100%, were achieved, making the product clinically useful due to reduced total dose required, and commercially attractive due to low losses of starting material.

TABLE 1

PEG-Insulin Microsphere Preparations

Sample	Core Loading%	Encapsulation Efficiency(%)
K5000-MS (40% initial loading)	28.3	70.8
K5000-MS (26% initial loading)	22.4	85.6
K5000-MS (11% initial loading)	10.3	93.5
F5000-MS (25.4% initial loading)	19.3	75.9
F5000-MS (10.7% initial loading)	11.5	107.2

In Vitro Release of PEG-Insulin from Microspheres.

A sample of 23.8mg F5000 PEG-insulin microspheres (12.8% drug content, PLGA 45:55; 0.15dl/g IV, acid end groups) was suspended in 1.5 ml phosphate buffered saline (pH 7.4, 0.02% sodium azide and 0.02% Tween20) and incubated at 37 C. The supernatant was withdrawn at intervals and analyzed by RP HPLC for released PEG-insulin. The buffer was replaced with fresh PBS and the incubation continued. The data were analyzed for cumulative release as a function of incubation time. The result is shown in Figure 1. Less than 10% of the PEG-insulin is released in the first day and 90% is released within 14 days. The low "burst" release, high total release and duration over a two-week period are highly desired features of a sustained release insulin formulation.

Other preparations made by the method of Example 9 using F5000 with different biodegradable polymers and also using K5000 PEG-Insulin gave one-day release values of between 0 and 7.5% and release durations up to 60 days.

In Vivo Pharmacodynamics and Pharmacokinetics for F5000 PEG-Insulin PLGA Microspheres.

F5000 PEG-insulin in microspheres composed of PLGA 45:55. 0.15dl/g IV, acid end groups (13.8% coreload) were tested for glucose suppression and for PEG-insulin pharmacokinetics in diabetic rats. Sprague-Dawley rats were treated with streptozotocin (Junod, A. et al., J. Clin. Invest., 48 (11): 2129-2139, 1969) to ablate their pancreatic B-cells 1 day before test article administration. Blood glucose and

PEG-insulin (ELISA assay) were measured pre-treatment and at various time intervals after subcutaneous injection of PEG-insulin microspheres at a dosage of approximately 125 IU/kg rat (~10mg F5000/kg). 4 diabetic rats were treated with the PEG-insulin microsphere formulation and 8 diabetic rats served as control subjects. Each rat's pre-treatment blood glucose level served as its own reference for 100% BGL (% basal glucose level). Figures 2 and 3 show the serum glucose levels and serum PEG-insulin levels over a 21-day period (data are means +/- SE).

Figure 2 shows that blood glucose levels are near 100% (within error of measurement) for the control diabetic rats throughout the experiment, as expected. For the PEG-insulin microsphere treated diabetic rats, the blood glucose level drops to around 50% of pretreatment levels and remain suppressed for 14 days. Figure 3 shows PEG-insulin levels rising on the first day post-treatment and remaining elevated until day 14 at which time they drop back to control levels. The dynamic effect (lowering of serum glucose levels) is measurable over 14 days and serum levels of F5000 are also measurable for 14 days. Thus, a pharmacokinetic/pharmacodynamic (PK/PD) correlation appears to exist for this example.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

What is claimed:

1. A method for preparing a protein-polymer conjugate comprising:
   (a) contacting an insulin protein with a hydrophilic polymer in the presence of at least one organic solvent and at least one metal chelator, under conditions that promote the formation of a conjugate of the protein and the polymer; and
   (b) isolating the conjugate.

2. The method of claim 1, wherein the insulin protein comprises human insulin.

3. The method of claim 1 or 2, wherein the hydrophilic polymer is selected from the group consisting of polyethylene glycol, polypropylene glycol, and linear, branched and amino-reactive derivatives thereof.

4. The method of claim 3, wherein the amino-reactive derivative is selected from the group consisting of an aldehyde, a N-hydroxy succinimide, a PNP-carbonate, and a benzotrizole terminated hydrophilic polymer.

5. The method of any of the preceding claims, wherein the hydrophilic polymer and insulin protein are contacted at a molar ratio of about 10:1-1:1.

6. The method of any of the preceding claims, wherein the organic solvent is selected from the group consisting of ethanol, methanol, DMSO, dioxane, DMF, and NMP.

7. The method of any of the preceding claims, wherein the organic solvent is present at a concentration of about 0.1 to 10%.

8. The method of any of the preceding claims, wherein the insulin protein and hydrophilic polymer are contacted at a protein concentration of about 0.1-5.0%.

9. The method of any of the preceding claims, wherein the insulin protein and hydrophilic polymer are contacted at a pH of about 5.0-7.5.

10. The method of any of the preceding claims, wherein the chelator is selected from the group consisting of polyvalent metal ion chelators, EDTA, deferoxamine (DEF), diethylenetriamine pentaacetic acid (DTPA), and bis(aminoethyl)glycolether N,N,N',N'–tetraacetic acid (EGTA).

11. The method of any of the preceding claims, wherein the chelator is present at a concentration of about 0.1-10 mM.

12. The method of any of the preceding claims, wherein the insulin protein and hydrophilic polymer are contacted at a temperature of about 4-50° C.

13. The method of any of the preceding claims, wherein the method further comprises the step of quenching formation of the conjugate prior to isolating the conjugate.

14. The method of claim 13, wherein the quenching is achieved by reducing the pH to about 1-4.

15. The method of any of the preceding claims, wherein the isolation is achieved by chromatography.

16. The method of claim 15, wherein the chromatography comprises ion exchange chromatography.

17. The method of any of the preceding claims, further comprising the step of encapsulating the conjugate in a biodegradable polymer.

18. A method for preparing an insulin-PEG conjugate comprising:

(a) contacting insulin with PEG in the presence of at least one organic solvent and at least one metal chelator, under conditions that promote the formation of a conjugate of the insulin and PEG; and

(b) isolating the conjugate.

19. The method of claim 18, wherein the insulin comprises human insulin.

20. The method of claim 18 or 19, wherein the PEG comprises an amino-reactive PEG derivative selected from the group consisting of an aldehyde, a N-hydroxy succinimide, a PNP-carbonate, and a benzotrizole terminated hydrophilic polymer.

21. The method of claim 18, 19, or 20, wherein the PEG and insulin are contacted at a molar ratio of about 10:1-1:1.

22. The method of claim 18, 19, 20, or 21, wherein the PEG and insulin are contacted at a protein concentration of about 0.1-5.0%.

23. The method of claim 18, 19, 20, 21 or 22, wherein the PEG and insulin are contacted at a pH of about 5.0-7.5.

24. The method of claim 18, 19, 20, 21, 22, or 23, further comprising the step of quenching formation of the conjugate prior to isolating the conjugate.

25. The method of claim 24, wherein the quenching is achieved by reducing the pH to about 1-4.

26. The method of claim 18, 19, 20, 21, 22, 23, 24 or 25, wherein the isolation is achieved by ion exchange chromatography.

27. The method of claim 18, 19, 20, 21, 22, 23, 24, 25 or 26, further comprising encapsulating the conjugate in a biodegradable polymer.

METHOD FOR PREPARATION OF SITE-SPECIFIC PROTEIN CONJUGATES

ABSTRACT

The invention is directed to a single-step method for rapidly and efficiently preparing protein-polymer conjugates, including an insulin-polymer conjugate. According to the method of the present invention, a protein and hydrophilic polymer are contacted in the presence of at least one organic solvent and at least one metal chelator, under conditions that promote the formation of a conjugate of the protein and polymer. Thus, the invention is directed to the site-specific modification of selected proteins, such as insulin, with poly(ethylene glycol) at residue PheB1. The invention also provides a pharmaceutical formulation for encapsulating the conjugate in a biodegradable polymer.

FIGURE 1

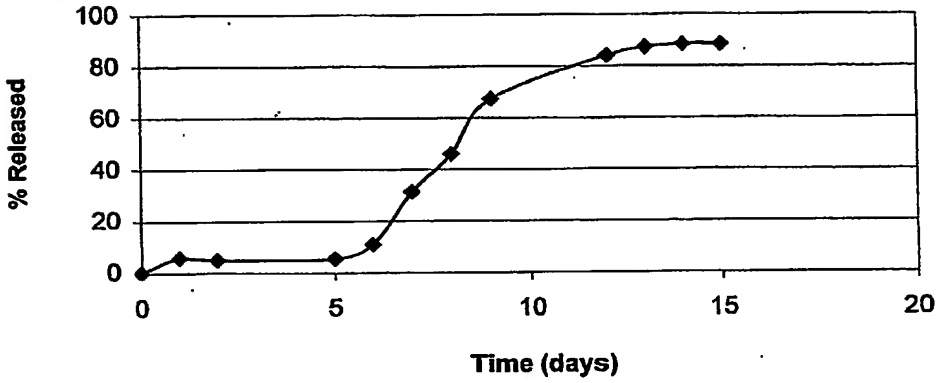

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FIGURE 2

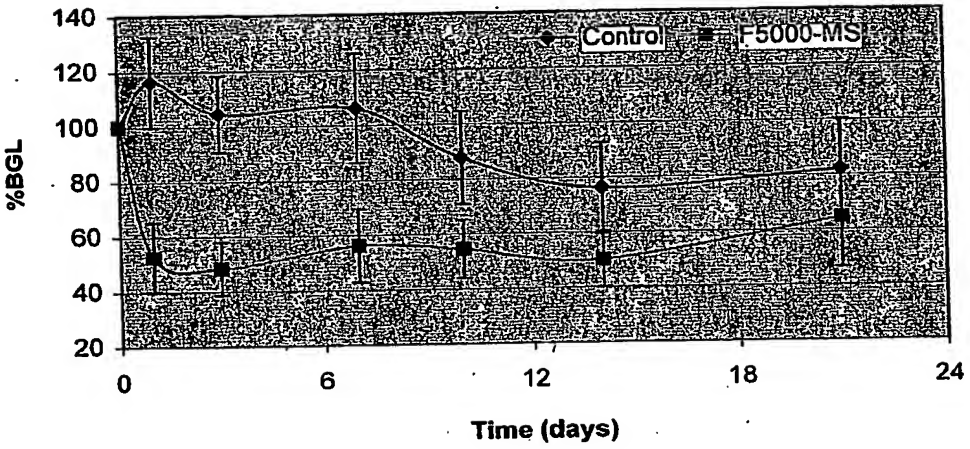

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FIGURE 3